UNCLASSIFIED

AD NUMBER

AD843842

NEW LIMITATION CHANGE

TO

Approved for public release, distribution unlimited

FROM

Distribution authorized to U.S. Gov't. agencies and their contractors; Foreign Government Information; JUL 1968. Other requests shall be referred to the Army Biological Laboratory, Attn: SMUFD-AE-T, Fort Detrick, MD 21701.

AUTHORITY

AMXFD, per ltr dtd 9 Feb 1972

THIS PAGE IS UNCLASSIFIED

(KL)

TRANSLATION NO. 1013

DATE: July 1968

DDC AVAILABILITY NOTICE

This document is subject to special export controls and each transmittal to foreign governments or foreign nationals may be made only with prior approval of Commanding Officer, Fort Detrick, ATTN: SMUFD-AE-T, Frederick, Md. 21701.

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

THE BACTERIAL FLORA OF THE TRACHEAE AND THE HEMOLYMPH OF SEVERAL INSECTS

[Following is translation of an article by Werner Pekl published in Zeitschrift fur Morphologie und Okologie der Tiere (Journal for Animal Morphology and Ecology), Vol 44, 1956, No 4, pp 442-458.]

A - Introduction

Although bacteriological investigations on insects are nearly as old as bacteriology itself -- Pasteur investigated silkworms, followed later by Metschnikoff and Paillot -- it has remained a little explored field of biological research. This is all the more surprising since bacteria in insects as carriers and hosts play a considerable role in medical entomology, in insect and plant pathology and even in the control of parasites.

The microbiological processes in the insect body are still not very well known. Not until Buchner and his school began the study of bacterial symbiosis of a number of insects did bacteria come again into the forefront of biological interests. Although the morphological side of the problem of symbiosis has been exhaustively illuminated by the intensive work of this school, physiological questions have begun to find a solution only very recently through the investigations of Aschner, of Glaser, of the school of Koch, and others.

However, the pure culture of symbiontic microbes, in particular that of the intracellular kind, still encounters great difficulties. The latter are absent for bacteria which live commensally with insects.

Since Stammer and others hold that symbiosis develops out of commensalism, investigation of the former is likely to be advanced through the study of such commensals.

Relatively little is known on the bacteriology of the hemolymph. So far about 250 different bacteria have been identified which occur in association with insects. This does not include the intracellular symbionts.

The greater part of the germs originated from the gastrointestinal tract but several researchers have isolated bacteria also from the blood of both diseased as well as healthy insects.

In 1931, Lilly observed that the blood of Musca Domestica may contain one or more species of bacteria even under completely normal conditions. He suspected that this flora of the hemolymph varies qualitatively and quantitatively with the age, nutrition and environment.

In 1942, Tauber and Griffith bred staphylococcus albus from the hemolymph of Blatta orientalis and Herms found the same germ in the blood of Phyllodromia germanica. This finding is in part understandable since, according to Metalnikov, staphylococcus albus is believed to be apathogenic for insects. Cameron frequently found bacteria in the blood of normal larvae and specifically for Graphiphora triangulum, Gonepteryx rhamni, Smerinthus ocellatus, Endromis versicolora, Graphiphora agathina, Ennomos autumnaria. Agrotis ashworthi, Euchloris vernaria and Aproophyta nigra. In 1934, the same author isolated several extremely virulent strains of Bacillus subtilis from the blood and the gastrointestinal tract of the wax moth. Artificially infected larvae died within 12-24 hours. However, other strains were shown to be apathogenic even at very high doses. The larvae survived the infection but spores persisted for a long time in the bodily cavities. Cameron also bred Streptococcus galleriae from the blood and the gastrointestinal tract of larvae of the was moth but this germ was also shown to be apathogenic.

Several reports on the occurrence of bacteria in the blood of diseased insects are available which go in part back to the time of Pasteur.

For example, Benedek and Specht (1933) found Bacillus megatherium, in addition to a fungus, in the hemolymph of diseased lecaniids. Giard and Billet, Inman, Issatschenko, Henneberg and many others described pathogenic glowing of insects through infection with photogenic bacteria (cf. Pfeiffer and Stammer). Particular mention should here be made of the studies of the latter on bacterium hemophosphoreum. They were able to induce septicemia through artificial infection and carry out interesting investigations on the cause of infection which covered both the behavior of the bacteria as well as the defensive reactions of the insect organism. Babers reported on septicemia produced by bacillus cereus in Prodonia cridania, Periplaneta americana and Plodia interpunctella. However, the same germ was also isolated from apparently entirely healthy insects (cf. Steinhaus).

Reports on the bacteriology of the tracheal system are not available in literature. We know that the respiratory tract of the higher animals is even under normal conditions host to germs which may become of importance under special circumstances.

Since the possible presence of microorganism in the tracheal system and in the blood is of great significance for many fields of research such as the breeding os symbionts from insect organs and in general for the orgin of many symbioses, it appeared necessary to carry out bacteriological investigation on the tracheal system and the hemolymph of different insects.

B - Methodology

1 - Sterile Preparations: Sterile preparation is probably the most difficult problem in bacteriological work with insects. Strict observation of sterile conditions is difficult already when working with larger subjects and the preparation of the very small areas involved in working with insects frequently is not at all simple. Some of the present bacteriological investigations of insects appear to be subject to caution in this respect.

In order to provide for sterile removement of tracheae and hemolymph in our experiments, we found it necessary first to test several of the customary practices of external disinfection and aseptic preparation in experiments with germ carriers by means of pellets of gypsum and siliceous earth which were coated with different cultures. This showed most of the methods to be very unreliable. Even if the subjects were dipped several times in alcohol and singed, some of the bacteriological controls always showed positive results. Prolonged immersion of the subjects in disinfectants such as absolute alcohol, hydrogen peroxide, alcoholic solution of chloramine and similar agents proved to be adverse because the agents diffuse into the tracheae and damaged or destroyed any germs existing here. Moreover, the prolonged interval created the risk of infection of the hemolymph by intestinal germs.

We found it best to dip the animal in hot melted paraffin, after celaning and surface coating with iodine (Note: I am indebted to Dr. Kellner of the Nuremberg Institute of Hygiene for suggesting this procedure). This kills off most of the surface germs and even very resistant spores (which would probably also have resisted any other treatment) were at least fixed and thus kept away from the field of operation. The germs of the internal organs including the tracheal system and the hemolymph did not seem to suffer any essential damage if the interval of immersion was not greatly prolonged.

After killing of the animals with ether, thorough cleaning and disinfection, they were prepared under sterile conditions. We first obtained specimens of hemolymph and then sections of tracheae and prepared cultures. When obtaining hemolymph, care must be exercised to prevent injury to the tracheae. In order to remove any adherent hemolymph from the tracheae, the latter were washed several times in sterile water. We prepared the abdomen ventrically for Gryllotalpa and dorsally for Dytiscus and Melolontha. For Apis, we prepared the particularly strongly developed tracheae of the anterior thorax which terminate in the first pair of stigmata. After corresponding preparation, we here obtain the lymph from the abdomen.

2 - Nutrient Media: Of the customary nutrient media, we utilized the following: blood plate, agar plate, Loffler plate, Endo plate, gelatin plate, potato wedge, neutralred agar, concentrated agar, and stab cultures of agar or of gelatin. Other cultures were prepared of nutrient bouillon, tryptophane bouillon, nitrate bouillon, aqueous peptone and milk. Generally, a diversified series was prepared from the following substances: glucose, lactose, saccarose, salicin, maltose, dulcite, mannite, xylose. More specific media were necessary only in some cases. As far as possible, we utilized "Bra [illegible]" Dry nutrient media which are characterized by constant composition. [illegible] was shown to be favorable to add an insect decoction, especially to the incubation media. In the preparation of the nutrient media as well as in diagnostic staining and the evaluation of metabolism, growth and stainability, we proceeded in accordance with the suggestions of the "Committee on Techniques of Bacteriological Investigation" of the American bacteriologists.

3 - Culture of Bacteria: The plates inoculated with tracheae or hemolymph were allowed to incubate either at room temperature or at 37° in the thermostat until they showed macroscopically plainly visible growth. If the colonies appeared uniform under the plate microscope, a smear was stained for Gram. If the microscopic picture was also uniform, fresh plates or oblique agar tubes were then inoculated by transfer. Mixed cultures were separated either by smears on plates or by the modified Koch effusion procedure.

4 - Determination of Pure Cultures: Pure cultures were determined in accordance with "Bergey's Manual" and "Bacteriological Diagnostics" by Lehmann-Neumann. As far as possible, we followed the more recent American publication. However, it was frequently not possible to follow the very rigid scheme of Bergey, especially in regard to the metabolic and physiologic characteristics of the incubated strains. Numerous variants manifested themselves so that it was necessary occasionally to draw primarily on the morphological characteristics also preferred by Lehmann-Neumann.

C - Our Own Bacteriological Investigation

I. Hemolymph and Tracheae

1 - Gryllotalpa: 100 animals were investigated with the technique indicated. Bacteria could be culturally demonstrated in the tracheae of 40 gryllotalpae. For 13 of these animals, a positive finding was also noted for the hemolymph. In one single animal, a positive finding was shown for the hemolymph (cf. Table 1 and 2) although the tracheal finding was negative.

Table 1

Insect Species	Total Numbered Examined	Negative Findings in Tra-cheae and Lymph	Positive Findings in Tracheae	Positive Findings in Tra-cheae and Lymph	Lymph Positive and Tra-cheae Neg-ative	Number of Cultures Isolated	Number of Different Germs
Gryllotalpa vulg..........	100	19	80	13	1	104	17
Melolontha mel. (series one)..	50	5	45	4	0	50	9
Melolontha mel. (series two)..	40	7	32	6	1	65	11
Dytiscus margi-nalis.........	8	1	7	2	0	12	6
Apis mellifica (healthy).....	100	16	83	4	1	103	15
Apis mellifica (m. Acarapis).	100	17	83	16	0	113	17
Apis mellifica (flight-disabled).....	50	7	43	3	0	53	12
Grand Total.....	448	72	373	48	3	505	36

Table 2

Germ	Tracheae		Lymph		Total	Surface
Actinomyces..............	7x	+	2x	+	9x	+
Bacillus cereus...........	2x	++	-	-	2x	-
Bacillus megatherium......	6x	+	-	-	6x	+
Bacillus mesentericus.....	7x	+	-	-	7x	+
Bacillus mycoides.........	10x	+++	3x	+	13x	+
Bacillus subtilis.........	15x	++	5x	+o. ++	20x	+
Bacillus vulgatus.........	4x	++	-	-	4x	-
Corynebacterium helvolum..	7x	++	1x	+	8x	-
Corynebacterium tumescens.	2x	++	-	-	2x	-
Escherichia coli..........	4x	++	-	-	4x	+
Micrococcus candicans.....	7x	+++	2x	+o. ++	9x	+
Micrococcus flavus........	2x	+	-	-	2x	-
Micrococcus pyogenes var. albus..................	6x	++	2x	+	8x	+
Micrococcus varians.......	5x	++o. +++	2x	+o. ++	7x	+
Proteus vulgaris..........	2x	+-	-	-	2x	-
Sarcina alba..............	5x	++	-	-	5x	+
Sarcina lutea.............	1x	++	-	-	1x	-
Grand Total...............	92x		17x		109x	

+- = very few; + = few; ++ = average; +++ = numerous.

The indications "average", "numerous" etc. as a measure of bacterial incidence are obviously very relative. It is scarcely possiblt to obtain here exact quantitative indications. Since smears were always obtained from several sections of tracheae and/or specimens of hemolymph, I calculated the average value per smear and established the following evaluation: 0-1 colonies = very little; 1-2 colonies = little; 2-5 colonies = average; 5 colonies and over = numerous.

In tracheae treated with bacterial stains, it was occasionally possible to demonstrate rods, cocci and spores. The incidence was always very minor (as already stated, such cultural indications as "average" or "numerous" are very relative). In general, the chitinized tracheae probably present most unfavorable media for germs so that they house perhaps mostly intermediate stages (e.g. spores).

As was to be expected, separate investigation of the strong anterior and the fine posterior sections of the tracheae showed that the large tracheal branches contain appreciably more bacteria than the delicate ramifications.

2 - Melolontha melolontha: We investigated animals captured in two different localities (series 1 near Stuttgart and series 2 near Heidelberg).

a) Among a total of 50 animals, 45 showed positive findings for the tracheae and 4 of these 45 also showed a positive finding for the hemolymph (table 1 and 3).

Table 3

Germ	Tracheae		Lymph		Total
Bacillus mesentericus..................	6x	+	1x	+	7x
Bacillus mycoides......................	3x	-o.+	-	-	3x
Bacillus subtilis......................	7x	+o.++	2x	+	9x
Corynebacterium simplex................	3x	+	-	-	3x
Flavobacterium diffusum................	5x	+	-	-	5x
Micrococcus flavus.....................	8x	+	-	-	8x
Micrococcus pyogenes var. albus........	3x	++	-	-	3x
Proteus vulgaris.......................	6x	-o.+	-	-	6x
Sarcina lutea..........................	5x	+	1x	++	6x
Grand Total............................	46x		4x		50x

b) A total of 40 animals was tested in this series. Thirty-two showed positive findings from tracheal cultures. Six of the animals showed findings for both tracheae and hemolymph. In one case the hemolymph was positive but the tracheae remained negative (table 1 and 4).

Table 4

Germ	Tracheae		Lymph		Total
Bacillus cereus........................	12x	+ to +++	3x	+o.++	15x
Bacillus megatherium...................	1x	+++	-	-	1x
Bacillus mesentericus..................	6x	++	-	-	6x
Bacillus subtilis......................	3x	+	1x	+	4x
Chromobacterium ianthinum..............	2x	+	-	-	2x
Escherichia coli.......................	5x	+	-	-	5x
Flavobacterium rhenanus................	2x	-o.+	-	-	2x
Micrococcus flavus.....................	9x	++	-	-	9x
Micrococcus pyogenes var. albus........	7x	++o.+++	2x	++	9x
Pseudomonas mildenbergii...............	1x	+	-	-	1x
Sarcina alba...........................	11x	+ to +++	-	-	11x
Grand Total............................	59x		6x		65x

3 - Epicaeus marginalis: Among a total of 8 subjects investigated, 2 showed bacteria in tracheae and hemolymph and in 5 only the tracheae were positive (table 1 and 5).

Table 5

Germ	Tracheae		Lymph		Total
Bacillus mesentericus..............	1x	++	-	-	1
Chromobacterium violaceum...........	2x	++	-	-	2
Micrococcus candicans..............	3x	+	-	-	3
Micrococcus pyogenes var. aureus....	2x	+++	2x	+	4
Micrococcus varians................	1x	+	-	-	1
Vibrio lique faciens...............	1x	+	-	-	1
Grand Total.......................	10x		2x		12

4 - Apis mellifica: We investigated three groups.

a) Healthy Bees: We tested 100 subjects, found bacteria from the tracheae in 83 and simultaneously in the hemolymph in 4 of the latter. One subject was positive for hemolymph and negative for tracheae (table 1 and 6).

Table 6

Germ	Tracheae		Lymph		Total	Environ-ment
Actinomyces........................	6x	+	-	-	6x	-
Bacillus alvei.....................	1x	+	-	-	1x	-
Bacillus mesentericus..............	8x	+	-	-	8x	-
Bacillus mycoides..................	9x	++	2x	+	11x	-
Bacillus subtilis..................	13x	+	2x	+-	15x	-
Bacterium prodigiosum..............	2x	+	-	-	2x	-
Micrococcus luteus.................	16x	+	1x	-	17x	-
Micrococcus luteus.................	8x	++	1x	+	9x	-
Micrococcus pyogenes var. albus.....	4x	+	1x	+	5x	-
Micrococcus radiatus...............	2x	+	-	-	2x	-
Proteus mirabilis..................	5x	-	-	-	5x	-
Pseudomonas fluorescens............	3x	+-	-	-	3x	-
Sarcina aurantiaca.................	10x	+	-	-	10x	-
Sarcina flava......................	5x	+	-	-	5x	-
Sarcina lutea......................	9x	+	-	-	9x	-
Grand Total.......................	101x		7x		108x	

Examination of the bacteria on the surface of the bee produced the finding already obtained by White that there is a relative absence of germs and a relative monotony of the surface flora exists. White found only 3 species (bacillus A, B. cyaneus, and micrococcus C) on healthy adult bees of normal hives. This apparent absence of bacteria is more than remarkable since the body of the bee is practically predestined to carry foreign bodies (e.g. pollen). Further investigation will be necessary to determine whether a relation exists to the bacterial inhibitors of bee honey observed by Dold. The surface flora of the bee was not further analyzed. However, the Bacterium cyaneum observed by White was not found by us.

b) Mite-Infected Bees: Among 100 subjects investigated, cultural investigation produced bacteria from the tracheae 83 times and from the hemolymph 16 times (table 1 and 7).

Table 7

Germ	Tracheae		Lymph		Total	Environment
Actinomyces	2x	+	1x	+	3x	+
Acrobactar cloacae	5x	++	-	-	5x	-
Bacillus alvei	8x	+o.++	2x	+	10x	+
Bacillus mesontericus	5x	+	-	+	5x	+
Bacillus mycoides	11x	+++	4x	++	15x	+
Bacillus subtilis	9x	++o.+++	4x	+	13x	+
Bacterium prodigiosum	4x	++	1x	++	5x	+
Escherichia coli	2x	++o.+++	1x	+	3x	-
Micrococcus flavus	10x	+	2x	+	12x	+
Micrococcus lutous	2x	+	-	-	2x	+
Proteus mirabilis	2x	++	-	-	2x	-
Pseudomonas fluorescens	4x	++o.+++	1x	++	5x	+
Sarcina aurantiaca	5x	+	1x	+	6x	+
Sarcina flava	5x	+	2x	+	7x	+
Sarcina lutea	2x	+	-	-	2x	+
Streptococcus faecalis	9x	++	3x	++	12x	+
Streptococcus liquefaciens	4x	+o.++	2x	+	6x	+
Grand Total	89x		24x		113x	

c) Flight-disabled bees without mites: We have exploited the results from 50 subjects originating from infected hives and unable to fly but not showing any mites in the tracheae. Positive findings for the tracheae were made in 43 cases and 3 of the same cases also showed bacteria in the hemolymph (table 1 and 8).

Table 6

Germ	Trachea		Lymph		Total	Environment
Actinomyces..........................	5x	+	-	-	5x	+
Bacillus alvei.......................	1x	+	-	-	1x	-
Bacillus mesentericus................	2x	++	-	-	2x	+
Bacillus mycoides....................	7x	++	2x	+	9x	+
Bacillus subtilis....................	6x	++	-	-	6x	+
Bacterium prodigiosum................	3x	+	1x	-	4x	-
Micrococcus luteus...................	4x	++	-	-	4x	+
Micrococcus pyogenes var. albus......	5x	++	2x	+	7x	+
Proteus mirabilis....................	1x	+	-	-	1x	-
Pseudomonas fluorescens..............	4x	+	1x	+	5x	+
Sarcina flava........................	5x	+	-	-	5x	+
Streptococcus liquefaciens...........	3x	-o.+	1x	+	3x	-
Grand Total..........................	46x		7x		53x	

Among 448 insects investigated, the following 36 germs were isolated and determined: Actinomyces (G, A_1, A_2, A_3); Aerobacter cloacae (A_2); Bacillus alvei (A_1, A_2, A_3); Bacillus cereus (G, A_2); Bacillus megatherium (G, A_2); Bacillus mesentericus; Bacillus mesentericus (G, M_1, M_2, D, A_2, A_3); Bacillus mycoides (G, M_1, A_1, A_2, A_3); Bacillus subtilis (G, M_1, M_2, A_1, A_2, A_3); Bacillus vulgatus (G); Bacterium prodigiosum (A_1, A_2, A_3); Chromobacterium ianthinum (A_2); Chromobacterium (D); Corynebacterium helvolum (G); Corynebacterium simplex (M_1); Corynebacterium [illegible]mescens (G); Escherichia coli (G, M_2, A_2); Flavobacterium diffusum (M_1); Flavobacterium rhenanus (M_2); Micrococcus candicans (G, D); Micrococcus flavus (G, M_1, M_2, A_1, A_2); Micrococcus luteus (A_1, A_2, A_3); Micrococcus pyogenes var. albus (G, M_1, M_2, A_1, A_2); Micrococcus pyogenes var. aureus (D); Micrococcus radiatus (A_1); Micrococcus varians (G, D); Proteus mirabilis (G, A_1, A_2, A_3); Proteus vulgaris (G, M_1); Pseudomonas fluorescens (A_1, A_2, A_3); Pseudomonas mildembergii (M_2); Sarcina alba (G, M_2); Sarcina aurantiaca (A_1, A_2); Sarcina flava (A_1, A_2, A_3); Sarcina lutea (G, M_1, A_1, A_2); Streptococcus faecalis (A_2); Streptococcus liquefaciens (A_2, A_3); Vibrio liquefaciens (D).

The letters in parentheses indicate the subjects in which the germs were found and their abbreviations stand for: G = Gryllotalpa; M_1 = Melolontha of series 1; M_2 = Melolontha of series 2; D = Dytiscus marginalis; A_1 = healthy bee; A_2 = mite-infected bee; A_3 = flight-disabled bees without tracheal mites.

II - Dependence of Tracheal Flora on Biotope

Considering the biotope of Gryllotalpa, typical soil germs were to be expected. It would be peculiar if the respiratory system of an insect continually moving in soil with a high germ content would be sterile. Especially since the tracheae must be regarded as an external environment displaced toward the interior. The tracheal flora varies qualitatively and quantitatively for each capture. Any degree of constancy could not be determined and an entirely different composition may result from renewed investigations.

A large part of the insects examined originated from the well fertilized beds of the Botanical Gardens. In order to examine the germ content of the corresponding area of habitation, soil samples were procured at a depth of about 10-15 centimeter and the number of germs determined by means of dilution and effusion procedures. It amounted to about 250,000,000 per gram of soil. We should remember here that such a procedure furnishes only a partial result.

We demonstrated individually Bacillus Subtilis, B. Mucoides, B. cereus, B. Mesentericus, Bacterium prodigiosum, various white and colored micrococci and sarcinae as well as actinomycetes. Approximately the same germs were obtained if insects were rubbed across the nutrient medium before having been cleaned (Table 2, column surface).

In order to test the difference with the germs of the air in the laboratory, air-plates were set out in the working space. We then found in particular white and colored micrococci, sarcinae, primarily Sarcina flava, once micrococcus pyog. bar. albus, once bacillus Mesentericus as well as a non-colored rod-shaped bacterium. Most of these germs had not been found at all or only to a minor extent in the soil.

The variety of tracheal flora therefore corresponds to that of the soil flora. Practically all isolated microorganisms represented typical and generally ubiquitous air and soil germs.

The findings obtained with gryllotalpae correspond entirely to those obtained from other insects. It was always possible to incubate a great number of bacteria from the tracheae and, for individual cases, also germs from the hemolymph. A particular constancy in the composition of the tracheal or the hemolymph flora was not found. Composition varied depending on and with the biotope. This was very marked in the series of melolontha which originated from different localities.

The findings for healthy bees also corresponded entirely to those just discussed.

III - Relation of Tracheal Flora to that of the Hemolymph

As already mentioned, several authors (Cameron, Lilly, Paillot, and others) were able to incubate bacteria from the hemolymph of apparently completely healthy insects. The origin of these micro-organisms and the reason for such a tolerance of the insects are still open questions.

It is notable in our investigations that only those bacteria were found in the blood which had also been demonstrated in the tracheae. A contamination of the hemolymph with tracheae germs was prevented by the technique of preparation utilized. An infection starting from the intestine can be excluded because most of the bacteria in the blood was not found in the intestine of the cricket either by Keller or by us. Although some species of bacteria (e.g. Bacillus Subtilis) were found both in the intestine as well as in the hemolymph, it was easily possible to differentiate the germs originating from the intestine from those of the hemolymph. An explanation of the interrelation of tracheal and hemolymph flora appears to be possible from the findings obtained through mite-infected bees.

In healthy bees, hemolymphatic germs are relatively infrequent according to our observations. The results from mite-infected bees were therefore all the more striking..

The bee mite (Acarapis woodi) is known to be located primarily in the especially strongly developed tracheae of the anterior thorax which terminate in the first pair of stigmata. The mite feeds on the blood of the bee which it ingests after puncturing the treacheal wall. With the injury to the tracheae, blood penetrates into the latter, coagulates and forms together with the defecation of the mite frequently an extended crust. It seemed most likely that this will also result in bacteriological consequences.

If we compare our findings from healthy bees with those of mite-infected bees, it is shown that the incidence in the tracheae is always much greater for the latter. The qualitative composition of the tracheal flora also varies with infection by mites. There then predominate proteolytes and/or saprophytes (in part pure fecal bacteria) and in part even strongly bee-pathogenic germs.

Even more striking are results for the hemolymph. The number of insects with bacteria in the hemolymph is three times greater with mite infection than the same number for healthy bees. The composition of the hemolymphatic flora here varied in the same sense as that of the tracheal flora so that the interrelation between the two here is especially notable. Especially for the mite-infected bees, the concept of an interrelation between the bacteria of the tracheae affected and the hemolymph offers no difficulties. It is also understandable that infection of the tracheae with mites and in particularly the formation of a crust leads to a qualitative and quantitative change of the bacterial flora.

IV - Experiments on Tracheal Infection

We now intended also to experimentally demonstrate the infection of the tracheae through the environment. For this purpose, we placed the insects in containers which had been contaminated with Bacillus Megatherium and staphylococcus albus (micrococcus pyogenes var. albus). In some cases, we also contaminated the surface of the insects, by avoiding the stigmata, with the germs which could be easily isolated by means of elective media. After the insects had remained for several days in the containers, they were prepared in customary manner and samples from the tracheae and the hemolymph were transferred to Bouillon cultures. After a brief period of incubation, one-half of the nutrient solution was mixed with liquified nutrient agar containing 10% sodium chloride (elective medium for staphylococcus albus) and formed into plates.

The other half was heated in the autoclave and formed into plates together with the nutrient agar containing 50% dextrose (elective medium for bacillus megatherium).

We first tested 12 gryllotalpae in this manner. The insects had remained three days in the infected containers. The result is shown in Table 9.

Table 9

Number of Insects	Tracheae		Lymph	
	Staphylococci	Bacilli	Staphylococci	Bacilli
3	0	0	0	0
2	+	+	0	+
1	+	+	+	0
5	+	+	0	0
1	0	+	0	0
12	8x	9x	1x	2x

The experiment was repeated with 20 healthy bees which remained for five days in the infected containers. (cf. Table 10). Table 11 shows the findings obtained with 20 mite-infected bees.

Table 10

Number of Insects	Tracheae		Lymph	
	Staphylococci	Bacilli	Staphylococci	Bacilli
6	0	0	0	0
7	+	+	0	+
1	0	+	0	+
3	+	+	0	0
1	+	0	0	0
2	0	+	0	0
20	11x	13x	0x	2x

Table 11

Number of Insects	Tracheae		Lymph	
	Staphylococci	Bacilli	Staphylococci	Bacilli
5	0	0	0	0
5	+	+	+	+
5	+	+	0	0
5	0	+	0	0
20	14x	17x	6x	6x

This clearly showed that environmental germs had access to the tracheae and that the possibility of an infection of the hemolymph by way of the tracheae also exists.

D - Discussion of Findings

It should not be surprising that fungi do not make an appearance in our investigations. For example, in the almost neutral reaction of the garden soil, the bacterial flora is known to predominate by far. Moreover, we utilize pure bacterial nutrient media with an alkaline reaction whereas fungi prefer an acid milieu. Furthermore, the incubation plate was observed for an only relatively short period (which is sufficient for bacteria) and any possible individual molds could not be differentiated from other impurities. In a bacteriological investigation of the tracheal system, the obligatory anaerobes could evidently be excluded a priori which appreciably facilitated the procedure. We also completely disregarded the obligatory autotrophic species, i.e. germs which proliferate only on nutrient media without any organically bonded nitrogen. For the same reason, we omitted a demonstration of species of virus, rickettesiae, etc.

It is likely that fewer actinomycetes were demonstrated than would correspond to their quantitative occurrence. In the opinion of many authors (Lieske, Meyer, Rippel), they form the main component of the microflora of the soil. However, because of their very slow growth, they appear on the culture plates only after a long interval. As already indicated above, we generally did not observe the same plate that long. The isolated actinomycetes were initially green, were firm and brittle and firmly integrated with the nutrient medium. They showed a characteristic smell of soil and strong hemolysis. After some time, they had a mat, chalky and dusty appearance. They were probably actinomyces odorifer or a closely related micro-organism.

The present investigations may be considered as having confirmed that the tracheae of the insects practically always contain germs. The tracheal infection is actually probably always 100% and the negative findings are probably due to the careful disinfection. The interrelation with the biotope is as striking as it is evident. Since the biotope generally contains many different germs, the tracheal flora is also generally rather variegated. This interrelation was very plain for gryllotalpae. The variegation of the germ flora of the soil is known. The latter constitutes an extremely non-homogenuous medium in which conditions change constantly not only with depth but even at the same level. Any mineral part: le of a different kind creats new environmental conditions. Any insect particle can form an alkaline microzone of changed reaction due to the formation of ammonia. Pieces of cellulose or lignine may shift reaction toward acid and create changed nutritional conditions. In the same way, living plant roots may lead to other conditions (Rippel-Baldes). Measurements with micro-methods have consequently shown different H-ion concentrations for different soil components. For the insects from beds of the botanical garden, human intervention is noticeable in the form of manure. After the addition of easily decomposing organic substances, we know that especially the bacteria decomposing protein and carbohydrates increase which can be regarded so-to-speak as "day laborers" (Rippel-Baldes). The typical representative of this group is bacillus mycoides. Grundmann has investigated the distribution of the latter at high altitudes where manured and non-manured soils often occur in close vicinity. In a similar manner, bacterium coli is the predominant form in water contaminated by fecal matter and does not prosper in pure water and non-contaminated soil. The seasonally and the climatically conditioned variation of the microorganism content of the soil may also play a minor role in such investigations (for example, the microorganism content of the soil runs parallel to the product of temperature and moisture). Similar circumstances are found in the environment of the other insects investigated.

It is more difficult to understand the occurrence of bacteria in the hemolymph, although this has been frequently observed and is probalby not unusual in insects. Zander is of the opinion that actually only the mouth can be considered the means of access since the surface is coated by an impenetrable cover of chitine. The same is the case with the tracheae so that the germs cannot enter by way of the lateral respiratory apertures.

Tauber found staphylococcus albus as a pathogenic germ in the hemolymph and also occupied himself with the problem of the path of infection by microorganisms in the blood. He is of the opinion that the insect comes into contact with infected animals after shedding of the skin when the exoskeleton is very soft and vulnerable. At that time the bacteria are able to penetrate the delicate skin actively or enter the body through small cracks in the surface.

Pfeiffer and Stammer were not able to produce infection with bacterium hemophosphoreum by feeding and also assumed that infection takes place through an injury of the skin, of the intestine or by insect bites. However, such injuries may occur probably even more easily for the delicate tracheae so that the latter must also be considered as a source of infection.

That insect bites and especially a tracheal parisitism can lead to infection is plainly shown by the results of the investigations of mite-infected bees.

Since Cameron, Lilly, and others as well as ourselves often found infected insects, such infections do not seem to be exceptions. The insect organism apparently comes relatively frequently in very close contact with the germs of its environment which may explain the resistance of those insects against many bacteria. It appears entirely possible that a symbiosis may develop out of such a resistance to commensal germs. Similar circumstances may intervene here which lead to the formation of symbiontic germs in the intestinal flora through the natural population of the intestine by the germ of the food ingested. With further progress of the mutual adaptation between micro-and macro-organisms, the latter apparently makes available mycetomata (cf. Stammer for details). Further investigations in this direction would appear to be desirable.

The bacterial content of tracheae and hemolymph must of course be taken into account for bacteriological work with insects, especially the breeding of symbionts. In bacteriological work with insects, care must be exercised not to damage or injure not only the intestine and intestinal processes but also the tracheae. If germs are to be incubated from organs, the germ content of the blood must be taken into account. It is recommended in any event in such cases to also incubate specimens of hemolymph and to wash the organs thoroughly in sterile water and possibly even to disinfect them. The probability of incubating extraneous germs for the insects is very high. Some allegedly incubated symbionts probably represent merely extraneous germs transplanted to the culture even when employing very careful techniques. Very strict requirements are here necessary in view of authors like Schanderl who have recently bred so-called "symbionts" from a great variety of animal and plant tissues. Such requirements should cover both experimental techniques as well as interpretation. Germs growing on general nutrient media should be regarded especially skeptically. According to Stammer, the following requirements must be satisfied for confirmation of a new symbiosis.

Demonstration of the uniformity of the occurrence of the symbionts at different developmental stages of the host, knowledge of the morphology and the change of form of the symbionts on the basis of microbiological staining methods and demonstration of the type of transfer of the symbionts to the progeny. As a single criterion, the bacterial culture is a much too complicated problem. As already stated, it may be difficult under certain circumstances to exclude extraneous germs and, on the other hand, many symbionts can not be bred with the customary bacteriological methods. For demonstration of the identity of the incubated bacteria with the symbiont, serological methods can be employed which has been pointed out by Gubler and Keller.

Summary

The authors report on bacteriological investigations of the tracheal system and the hemolymph of various insects as well as on experimental infection. The results may be summarized as follows.

1. The tracheae of the insects contain bacteria which are generally the same as those of the environment.

2. A population with environmental germs can be produced experimentally.

3. The hemolymph may also contain bacteria without observable disease symptoms in the insect.

4. The bacterial content of tracheae and hemolymph is increased when the tracheae are infected with parasitic mites.

5. An interrelation seems to exists between the bacteria of the tracheae and of the hemolymph which is manifested especially clearly in treacheal parasitism whereas the germs found in the hemolymph generally do not show any clear interrelation with those of the intestinal flora.

6. The particular significance of the bacterial content of tracheae and hemolymph for research on symbiosis is pointed out in conclusion.

Literature References

Armbruster, L.: On the problem of Acarapis. Arch. Bienenkde 7, 313-327 (1926).

Aschner, M., and E. Ries: The Behavior of the Body Louse When Eliminating its Symbionts. Z. Morph. u. Okol. Tiere 26, 529-590 (1932).

Babers, F. H.: Septicemia of the southern army worm, causes by Bacillus cereus. Ann. Entomol. Soc. Amer. 31, 371-373 (1938).

Baumgartel, Tr.: Microbic Symbioses. Jena, 1942. - Microbic Symbioses in the Plant and Animal Kingdom. Wissenschaft 94 (1946).

Benedek, T., and G. Specht: Micrologic-Bacteriologic Investigations on Fungi and Bacteria as Symbionts in insects. Zbl. Bakter. I. Orig. 130, 74-90 (1933).

Bergey's: Manual of determinativ bacteriology, 6. Aufl. Baltimore 1948.

Borchert, A.: Epizootic Diseases of the Honeybee. Berlin, 1926. - Investigations of the Acarapis Mite. Z. Parasitenkde 4, 351-366 (1932). - On the Acarapis Mite and on the Problem of Control. Z. Parasitenkde. 12, 86-93 (1940).

Brown, F. M.: Descriptions of new bacterias found in insects. Am. Mus. Novitates 251, 11 (1927).

Brun ch* , K.: From the Respiratory Mechanism of Insects, in Particularly that of the Bees. Arch. Bienenkde 3, 154-160 (1922).

Buchner, P.: Animal and Plant in Symbiosis, 2. Aufl. Berlin 1930. - Symbiosis of Animals with Plant Micro-Organisms, 2. Aufl. Berlin, 1949. - Endosymbiosis of Animals with Plant Micro-Organisms. Basel and Stuttgart 1953.

Cameron, G. R.: Inflammation of the caterpilliars of Lepidoptera. J. of Path. 38, 441-466 (1934). Zit. nach Steinhaus.

Christenson, H. R.: Soil Composition and Bacteria. Zbl. Bakter. 11 Orig. 59, 266 (1923).

Dfegener, P.: The Tracheal System of Insects "Schroeder's Handbook of Entomology. Jena 1928.

Den Dooren de Jong: Studies on Bacteriology. Zbl. Bakter. 120, 1-23.

Dorner, W.: Methods for the Investigation of Bacterial Cultures. (Manual of pure culture study of bakterias) Hannover 1933.

Dreher, K.: Structure and Development of the Respiratory System of the Honeybee. Z. Morph. u. Okol. Tiere 31, 608-672 (1936).

Fink, R.: Morphological and Physiological Investigations of the Intracellular Symbionts of Pseudococcus citri. Z. Morph. u. Okol. Tiere 41, 78-146 (1952).

Gottheil, O.: Systematic Investigations on the Bacteria of the Soil Under Cultivation. Zbl. Bakter. 11 7, 589-633 (1901).

Grabner, K. E.: Comparative Morphological and Physiological Studies on the Symbionts of Anobiides and Cerambycides. Z. Morph. u. Okol. Tiere 42, 472-528 (1954).

Gubler, H. U.: Experiments on the Incubation of Intracellular Symbionts of Insects. Diss. Zurich 1947.

Hunkeler, A.: Investigations on the Intestinal Flora of the Honeybee. Diss. Zurich 1925.

Janke, A.: Working Methods of Microbiology. Dresden and Leipzig 1946.

Koller, H.: Bacteriological Investigations of the Intestinal Flora of the Cricket and of Intracellular Bacteria of Pelomyxa paluseris Greef. Diss. Erlangen 1949.

* Fifth letter illegible.

Koch, A.: On the occurrence of Mitochondria in Mycetocytes. Z. Morph and Okol. Tiere 19, 259-290 (1930). - On the Behavior of Larvae of Sitodrepa not housing Symbionts. Biol. Zbl. 55, 159-[illegible] (1935). - Fifty Years of Research on the Symbioses of Insects. Naturwiss. 37, 313-317 (1950).

Lehmann, K. B. and R. O. Neumann: Bacteriological Diagnostics. Munchen 1926/27.

Lieske, R.: Strahlenpilze (Actinomycetes). Mikrokosmos 39, 10, 220 (1950).

Lengerken, H. v.: The Animal Kingdom. Bd. IV/3 Insekten. Berlin 1953.

Lilly, J. H.: A preliminary study of the presence of bacteria in the blood of house-fly. University of Wisconsin. B. S. Thesis. 1931. Zit. nach Steinhaus.

Maasson, A.. and H. Bohn: Bacteriological Soil Investigations. Zbl. Bakter. II Orig. 62, 180 (1939).

Marcu, O.: A Contribution to the Knowledge of Tracheae. Zool. Anz. 89, 136-139 (1930).

McCray, A. H. and G. F. White: The diagnosis of bee diseases by laboratory methods. U.S. Dep. Agric., Bull. No 671, 1918.

Metalnikov, S.: Micro-Infection and Immunity in the Mite of Apis Galleria Mellonella. Monogr. Inst. Pasteur. Paris 1927.

Meyer, R.: Micro-Biological Practice. Wolfenbuttel and Hannover 1948. - The Role of Micro-Organisms in the Human Regime of the Soil. Mikrokosmos 40, 3, 57 (1950).

Meyn, A.: Determinative Index for Genus Classification of Bacteria. Stuttgart 1952.

Morgenthaler, O.: A Decade of Mite Disease of the Honeybee. Z. angew. Entomol. 19, H. 3 (1932).

Muller, R.: Medicinische Mikrobiologie. Munchen and Berlin 1950.

Paillot, A.: Infection Among Insects. Trevoux 1933. Zit. nach Steinhaus.

Pfeiffer, H. and H. J. Stammer: Pathogenic Phosphorescence in Insects. Z. Morph. u. Okol. Tiere 20, 136-171 (1930).

Prell, H.: Contribution to the Knowledge of the Mite Infection of the Honeybee. Arch. Bienenkde 8, 241-275 (1927).

Rippel-Baldes, A.: Basis of Micro-Biology. Berlin and Gottingen 1947.

Rokitzka, A.: General Micro-Biology. Munchen 1949.

Sachs, H. G.: Enemy No. One of the Bee. Mikrokosmos 42, 121-124, 240-251 (1953).

Schanderl, H.: Botanic Bacteriology and Nitrogen Metabolism of Plants on a New Basis. Stuttgart 1947. - On the Yeast Symbiosis of Cerambycides and Aphites. Verh. dtsch. Zool. 1950, 252-256.

Schonichen, W.: Practice of Entomology. Jena 1930.

Stammer, H. J.: The Distribution of Endosymbiosis in Insects. Tijdschr. Entomol. Deel 95, 23-40 (1952).

Steinhaus, E. A.: Insect Microbiology. New York 1946. - Principles of Insect Pathology. New York 1949.

Tauber, O. E., and J. T. Griffiths: Isolation of Staphylococcus albus from hemolymph of the roach Blatta orientalis. Proc. Soc. Exper. Biol. a. Med. 51, 45-47 (1942). Zit. nach Steinhaus.

Wallenfels, K.: Symbiosis and Antibiosis. Chemie 1945, 1-16.

Weber, H.: Handbook of Entomology. Jena 1933.

White, G. F.: The bacteria of the apiary with special reference to bee diseases. U. S. Dept. Agric. Bull. v. Entomol. Techn. Bull. 1906, 14.

White, G. F., and P. Bruce: The normal bacterial flora of the bee. Aberdeen 1921.

Wohlgemuth, O. E.: The Respiratory Stigmata of the Honeybee. Diss. Erlangen 1929.

Zander, E.: The Structure of the Bee. Stuttgart 1946. - Diseases and Parasites of the Adult Bee. Stuttgart 1947